AD_____

Award Number: W81XWH-10-1-0941

TITLE: Spinal Cord Repair with Engineered Nervous Tissue

PRINCIPAL INVESTIGATOR: Douglas H. Smith, M.D.

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia PA 19104

REPORT DATE: October 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2012	Annual	30 September 2011-29 September 2012

4. TITLE AND SUBTITLE
Spinal Cord Repair with Engineered Nervous Tissue

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0941

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
DOUGLAS H. SMITH, MD

E-Mail: smithdou@mail.med.upenn.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pennsylvania
PHILADELPHIA PA 19104

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
. Spinal cord injury (SCI) exacts an enormous social and financial burden on society. As such, there has been considerable attention directed at finding treatment strategies, including development of tissue and cell transplant techniques. However, the current approaches do not adequately address the complexity of the injury site, such as lesion length and an environment hat is usually non-permissive for axon regeneration. We have developed tissue engineered constructs consisting of living dorsal root anglia (DRG) and axons that can be stretch-grown to a length necessary to bridge extensive lesions. In current studies, we have optimized in vivo laminoplasty procedures, transplanted stretch-grown constructs into a 1cm-long lesion in the rat thoracic spinal cord, and have demonstrated survival of DRGs up to 1month post-implantation. We intend to evaluate long-term (3 and 6 month) survival of the constructs as well as functional recovery beyond the lesion site. If successful, this approach will provide an alternative or additional means to repair large spinal lesions.

15. SUBJECT TERMS- n/a

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	12	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS

Page

INTRODUCTION

In 2012, the National Spinal Cord Injury Statistical Center (NSCISC) reported that there were approximately 40 cases per million population in the United States, or approximately 12,000 new cases, of spinal cord injury (SCI) each year[1]. In addition to these new cases, approximately 270,000 people in the United States live with a chronic SCI[1]. SCIs have become a common consequence of motor vehicle crashes, falls, and acts of violence (most typically gunshot wounds). Prior to receiving funding for this grant, our research group established proof-of-concept success of bridging a lateral hemisection in the rat spinal cord with engineered ("stretch-grown") living nervous tissue constructs[2]. For the current effort, we have developed a new model of a 1 cm long complete evacuation of the thoracic spinal cord. With this new model, we have initiated studies to 1) examine functional recovery following implantation of the living constructs bridging SCI lesion, and 2) to determine potential formation of new intraspinal circuits across the lesion, such as growth of host axons through the construct and synapse formation with neurons on the other side. Our first year was dedicated to hiring and training new personnel, developing and characterizing a new model of SCI and initiating our transplantation studies. This second year we have improved upon the laminoplasty technique used by providing extradural protection that minimizes connective tissue infiltration and compression of the transplanted constructs. Using this new method, we have observed improved neuronal survival and axonal projections out to 1 month post-implantation.

BODY

Specific Aim 1: Evaluation of effects of transplanted nervous tissue constructs on recovery of function over 6 months post-injury in a model of complete spinal cord segment excision (T9-T11).

We continue to transplant engineered living nerve constructs consisting of long axonal tracts (1 cm long) to bridge an excised segment (also 1 cm in length) of the rat thoracic spinal cord 10 days after injury. Groups include sham (surgery, no injury), injured with hydrogel insertion into the cavity/ lesion, and injured with the living construct embedded in hydrogel transplanted into the cavity. Once we have seen histological analyses supporting the formation of intraspinal circuits across the lesion, we will begin to perform weekly functional outcome assessments using a battery of tests to evaluate potential recovery of both motor and sensory function.

Specific Aim 2: Evaluation of the survival and integration of transplanted living nervous tissue constructs and host axon regeneration through the construct at 1, 3, and 6 months post transplantation. Using the same animals/groups from Aim 1, we have performed extensive histological examinations on constructs at 1 month post-implantation. As described in our 2011 Annual Report we had established the general laminoplasty procedure however we were encountering connective tissue infiltration into the vertebral column. Additionally, constructs suffered physical compression from the closure method and cell viability was affected. We addressed these concerns this year by incorporating a more sufficient extradural barrier into our surgical procedure in order to physically block connective tissue invasion and protect against compression. This barrier includes a layer of Teflon tape, followed by a rectangular piece of Gelfoam® (approximately 1.2 cm x 2-3 mm x 7 mm), sealed with 2 drops of Vetbond™ Tissue Adhesive (n-butyl cyanoacrylate). In addition, we have reformulated the hydrogel composition in order to insure higher viability and health of the DRG neurons once implanted. Furthermore, we have assessed the use of a ½ tube cover (either intradurally or extradurally) to protect the construct from both connective tissue infiltration and physical compression. For the assessment of the ½ tube cover we performed acute surgeries (constructs are implanted immediately following cord evacuation) on several animals to expedite the evaluation of these new methods of protection. To this end, we have performed histological analyses on DRG explants only (Figure 1) and stretch-grown constructs (Figure 2) implanted with ½ tubes (at 7 days and 12 days post-implantation, intradurally, and extradurally, respectively) in order to determine the viability of the DRGs and the constructs. Additionally, we evaluated the use of an extradural ½ tube cover for the protection of 1 cm stretch-grown constructs at 1 month post-implantation (Figures 3 and 4). These figures demonstrate the advantage to using this method as DRG bodies and axons remain viable and healthy at 1 month *in vivo*. For these

studies, we needed to determine that the constructs survived for at least 1 month before studying long-term outcomes (3 and 6 months).

PITFALLS: Currently, we have inconclusive histological identification of host vs. graft and have implemented GFP-transgenic rats to facilitate identification.

To date, despite complete removal of spinal cord, we have found that the constructs survive for at least one month after transplant and maintain their oriented geometry of long axon tracts. With these promising findings, we propose to examine functional recovery effects with long-term survival as well as potential penetration of host axons completely through spinal cord lesions to form new intraspinal circuits.

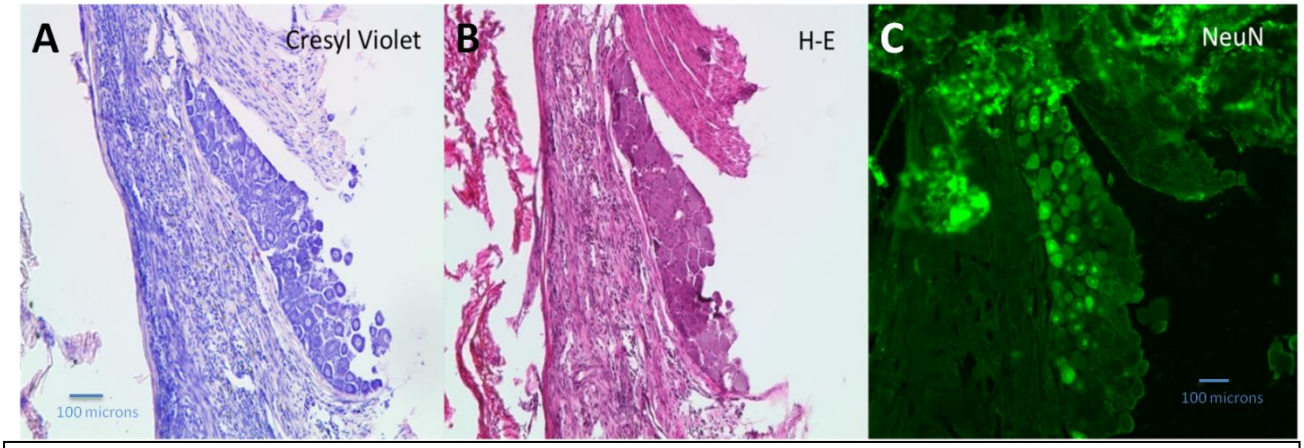

Figure 1: Proof of Protectiveness of ½ Tube Intradural Cover - DRG Explants Only at 7 days post-implantation
DRG explants harvested from E16 pups were suspended in a collagen hydrogel and implanted into the 1 cm cavity site. A ½ tube was used as an intradural cover in order to protect against infiltrating tissue and compression of the samples. At 7 days post-implantation, cells were evaluated via Cresyl Violet (**A**), Hematoxylin and Eosin (H&E) (**B**), and NeuN (neuronal nuclei) visualized with Alexa 488 secondary (green). Cells appeared to be circular, healthy, and tightly clustered.

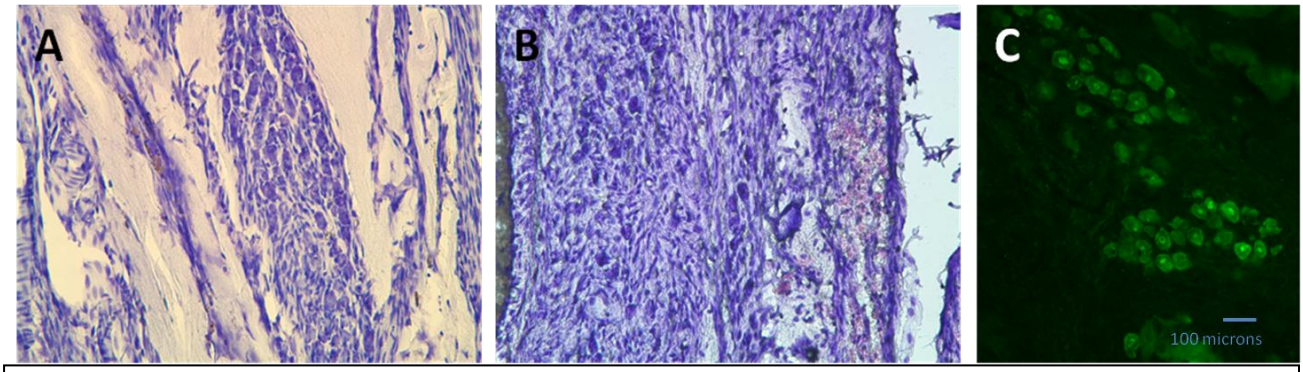

Figure 2: 1 cm Stretch-Grown Constructs With and Without a ½ Tube Extradural Cover. Construct stained with Cresyl Violet at 12 days post-implantation with (**A**) and without (**B**) a ½ tube extradural cover. In the transplant with a ½ tube cover (A), DRGs appear to be tightly clustered as compared to DRGs without the ½ tube extradural cover (B) that appear to be dispersed. **(C)** One month survival of a stretch-grown construct implanted with a ½ tube extradural cover (cell bodies identified by NeuN (neuronal nuclei) visualized with Alexa 488 secondary (green), 20X). Cell bodies appear circular and display a healthy morphology. Taken together, these findings support the use of an extradural ½ tube cover to provide increased protection of the graft area.

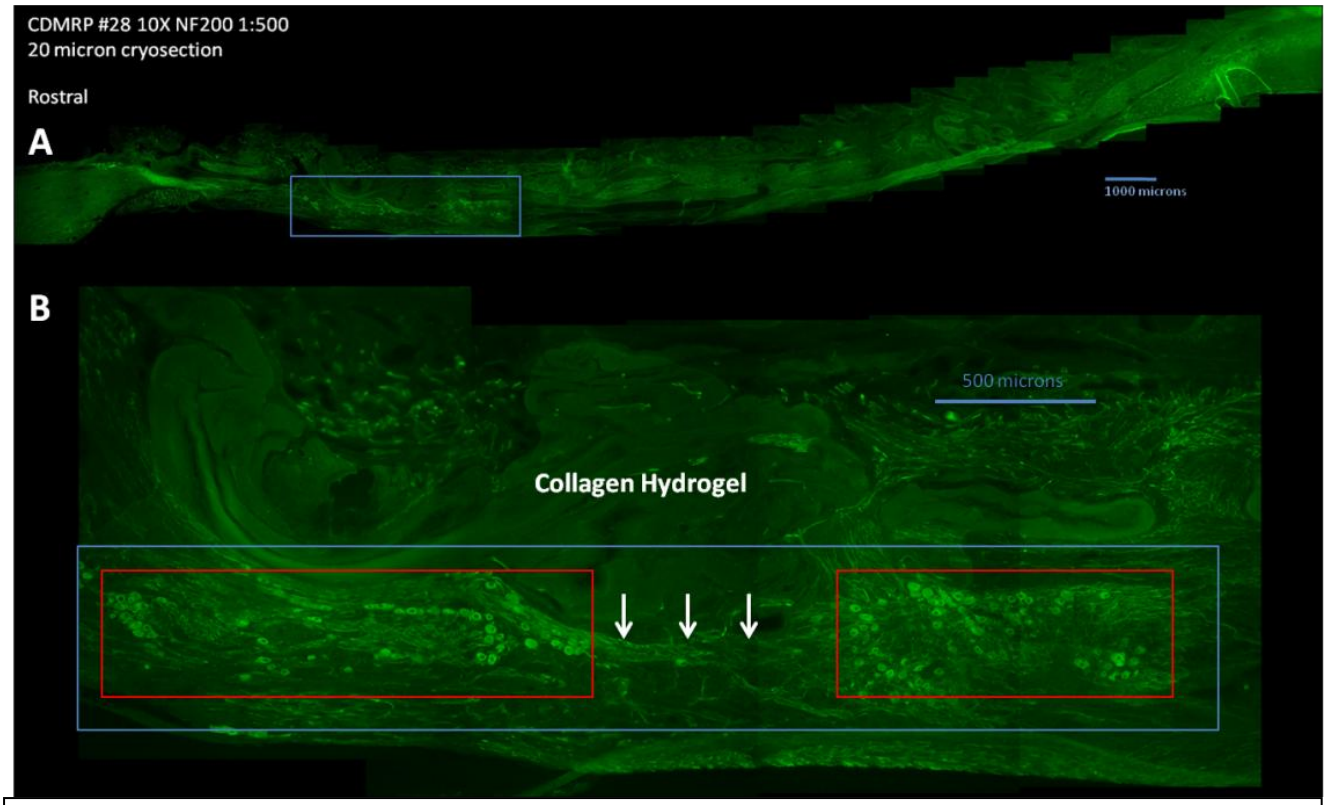

Figure 3: One month survival of DRG nerve construct protected by extradural ½ tube cover. Collagen hydrogel is intradural. Full length 1cm implantation site (**A**) in spinal cord. Blue rectangle denotes location of stretch-grown construct. (B) Magnified construct region showing transplanted DRG bodies (two opposing clusters shown within red boxes) and surviving axons (arrows) identified with NF200 visualized with Alexa 488 secondary (green). Axons remain intact and present between DRG clusters.

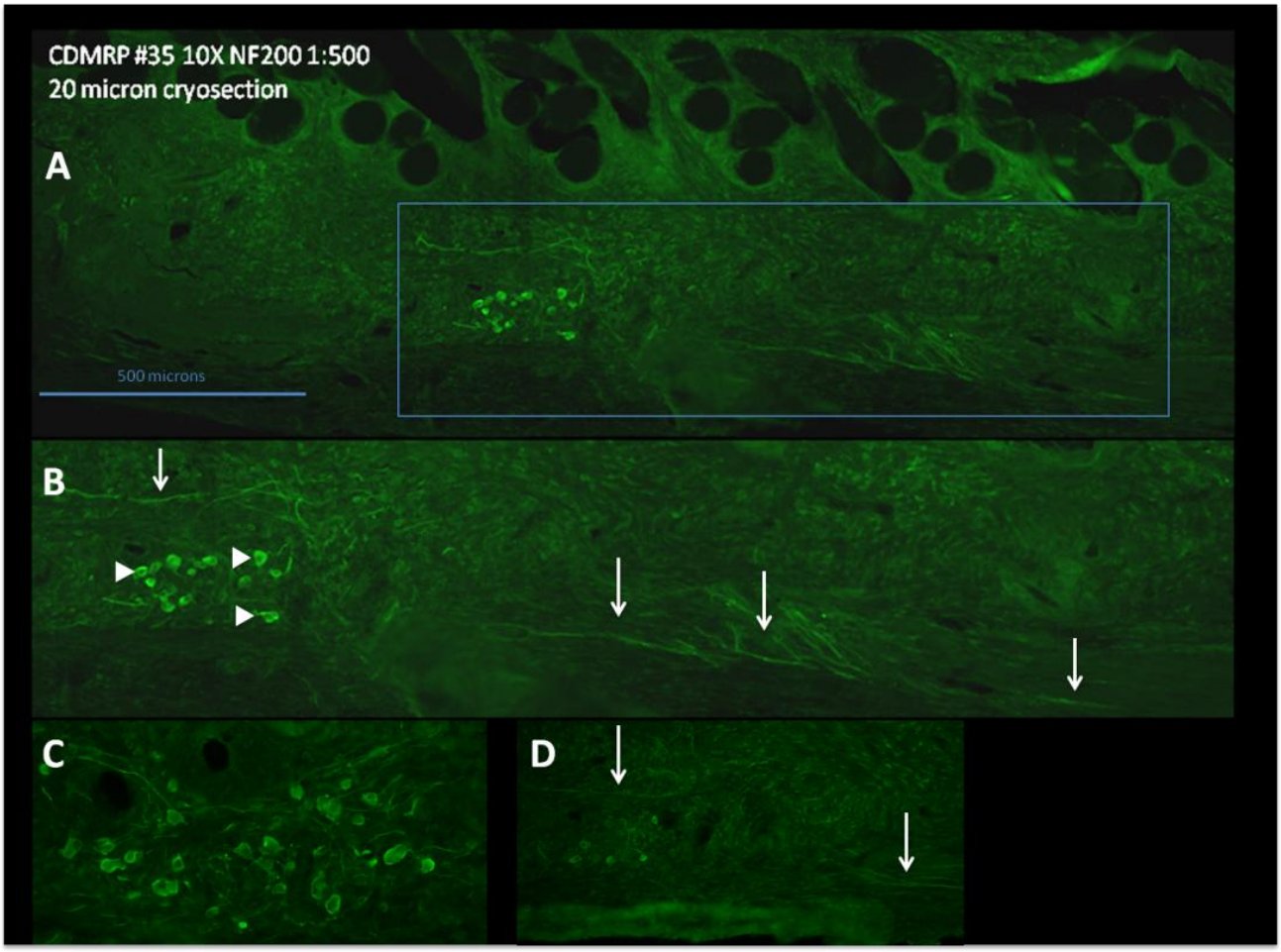

Figure 4: One month survival of DRG nerve construct protected by extradural ½ tube cover. (**A**) Implantation site in spinal cord. Blue rectangle denotes location of stretch-grown construct. (**B**) Magnified construct region shows transplanted DRG bodies (arrow heads) and surviving axons (arrows) identified with NF200 visualized with Alexa 488 secondary (green). Axons remain intact and extend from DRG bodies. (**C, D**) 20 μm anterior and posterior, respectively, to section B. Healthy, circular cell bodies (C) and axons (D, arrows) are present in sections lateral to section B.

KEY RESEARCH ACCOMPLISHMENTS

SPECIFIC AIM 1: Evaluation of effects of transplanted nervous tissue constructs on recovery of function over 6 months post-injury in a model of complete spinal cord segment excision (T9-T11).

• TASK: Construct transplantation. We have successfully transplanted several 1 cm stretch-grown constructs into animals for 1-month post-transplantation end points and survival. We have gathered histological analyses of these samples and are preparing for our next cohort of experiments in order to transplant constructs that will be maintained for 3 and 6 months post-transplantation end points and survival.

SPECIFIC AIM 2: Evaluation of the survival and integration of transplanted living nervous tissue constructs, and host axon regeneration through the construct at 1, 3, and 6 months post-transplantation.

• TASK: Tissue harvest and histological analyses: We have demonstrated that transplanted constructs survive 1 month post-surgery. We are in the process of generating animals that will be sacrificed at the appropriate 1, 3, and 6 months post-transplantation endpoints and processed.

• TASK: Compression Protection: We have demonstrated the effectiveness of our new closure method featuring a tri-layered barrier system consisting of a layer of Teflon tape, followed by a rectangular piece of Gelfoam®, sealed with 2 drops of Vetbond™ Tissue Adhesive (n-butyl cyanoacrylate). Additionally, we have evaluated the use of a ½ tube extradural cover that provides enhanced protection against tissue infiltration and physical compression resulting in higher viability and health of the transplanted cells.

REPORTABLE OUTCOMES

Due to the development of a new closure method to prevent connective tissue infiltration and physical compression of the stretch-grown constructs, in order to enhance cell viability and overall health, we have no published reports to note.

CONCLUSION

Over the course of this second year of funding, we have successfully developed a new closure method to our laminoplasty that reduces connective tissue infiltration and physical compression of our stretch-grown constructs within the spinal cord cavity so as to enhance survival and health of the constructs. Notably, this 1cm evacuation of the spinal cord both creates a cavity for transplant of our living tissue-engineered nerve construct and is of the scale lesions often encountered after SCI in humans. As an essential step for ongoing long-term studies, our data thus far demonstrate that the engineered nervous tissue constructs survive for at least one month after transplantation in this large lesion. One of the obstacles we encountered in our study is distinguishing host from transplanted tissue. We are currently addressing this issue by using histological identification of host vs. graft and have implemented additional GFP-transgenic rats to facilitate identification. Success of our studies will provide another avenue to bridging extensive SCI lesions to restore function.

REFERENCES

1. Spinal Cord Injury Facts and Figures at a Glance Fact Sheet. National Spinal Cord Injury Statistical Center February, 2012. Accessed 10/23/2012. https://www.nscisc.uab.edu.
2. Iwata A, Browne KD, Pfister BJ, Gruner JA, Smith DH. Long-term survival and outgrowth of mechanically engineered nervous tissue constructs implanted into spinal cord lesions. Tissue Eng. 2006;12(1):101-110.

APPENDICES

PERSONNEL SUPPORT PROVIDED BY THE AWARD

1. Douglas Smith
2. Kevin Browne
3. Timur Litvinov
4. Amy Kim
5. Mindy Ezra